Outcome of Patients with Non-Hodgkin's Lymphoma: Correlation with Apoptotic Markers

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ABSTRACT

Abnormalities in specific cell cycle control genes as B-cell lymphoma-2 (Bcl-2) family proteins and regulator of apoptosis as Fas/APO-1(CD95) commonly found in lymphoid neoplasm. The study aims to identify factors that can help in predicting the behavior of non-Hodgkin's lymphoma, selecting patients with higher response to different chemotherapeutic modalities and early detection of recurrence.

Thirty newly diagnosed Non-Hodgkin's lymphoma patients before treatment (group I), after receiving 4 cycles of chemotherapy (group II) and ten apparently healthy volunteers were chosen as controls (group III) were included in the study. All groups were studied for presence of Bcl-2 and human Fas/APO-1by ELISA.

Bcl-2 is significantly higher ($p<0.001$) in patients groups I and II compared to group III. Also, Bcl-2 serum levels of patients after treatment (group II) had highly significant reduction ($p<0.001$) comparable to group I. In addition, Fas/APO-1 represents significantly higher values in patients groups I and II compared to group III ($p< 0.001$) with statistically significant elevation among patients who attained complete remission than in those with partial or no remission after treatment.

It could be concluded that the elevated levels of Bcl-2 in NHL patients has been contributed to its role as a major anti-apoptotic factor in tumor genesis. However, its significant reduction after chemotherapy indicates that function of Bcl-2 is dependent upon which other members of the Bcl-2 family that is involved in controlling the upstream and downstream events. While Fas/APO-1 could be considered as an important apoptotic marker in NHL and can be considered as a good predictor for response to chemotherapy.

Key Words: NHL – Apoposis – Bcl-2 – CD95.

INTRODUCTION

Apoptosis is an intrinsic cell death program that plays critical roles in tissue homeostasis, especially in organs where high rates of daily cell production are offset by rapid cell turnover [1]. The hematopoietic system provides numerous examples confirming the importance of cell death mechanisms for achieving homeostatic control. It is characterized by a series of morphological and biochemical changes [2,3].

Non-Hodgkin's lymphomas are heterogeneous group of disorders with different clinical characteristics that usually relapsed and became difficult to control. Therefore, identification of factors that can help in predicting the behavior of the disease and selecting patients with higher response to different chemotherapeutic modalities or detection of recurrence at earlier stages before clinical manifestations is important [4-9].

B-cell lymphoma-2 (Bcl-2) family protein is a critical regulator of apoptosis, whose expression frequently becomes altered in human cancers, including lymphoma. Bcl-2 was the first member to be identified by virtue of its involvement in chromosomal translocation t (14:18), commonly found in B-cell non-Hodgkin's lymphoma [10-12].

An important regulator of apoptosis is Fas/APO-1 (CD95) which is a membrane glycoprotein belonging to the tumor necrosis factor/ nerve growth factor receptor family, which can trigger apoptosis in some lymphoid cell lines [1,13-16].

PATIENTS AND METHODS

This study was performed in Clinical Hematology Unit of Internal Medicine Department,

Assiut University Hospitals. The study included thirty patients (22 males and 8 females) with Non Hodgkin's Lymphoma. Their mean age was 42±7.13 years. Those newly diagnosed before receiving radio or chemotherapy were considered as group (I). The same patients after receiving 4 cycles of chemotherapy were considered as group (II). Ten apparently healthy volunteers were chosen as a control group (III) and they were age and sex matched to the patients.

Inclusion criteria for patients with NHL (group I):

Patients newly diagnosed with documented histopathological diagnosis of NHL who were not receiving any radio or chemotherapy.

Exclusion criteria of patients with NHL:

1- Patients with NHL who previously received steroids, chemotherapy or recombinant cytokine therapy.

2- Patients with NHL in relapse.

All patients were subjected to full history taking, thorough clinical evaluation, baseline investigations and specific investigations for detection of Bcl-2 protein and human FAS/APO-1 using ELISA technique.

All patients were treated with (CVP) regimen for low grade and (CHOP) chemotherapy regimen for intermediate and high grade lymphoma. They were subdivided according to the response of treatment into those acquired complete remission (CR): 12 patients, partial remission (PR) 4 patients, no remission (NR) 12 patients according to Banadonna [17]. Two patients were defaulters before completing 4 cycles of chemotherapy.

Statistical analysis:

The data of each group are tabulated in the Microsoft Excel 97 program from the master sheet giving a serial number for each subject, then expressed as mean ± SE for all parameters. The data were analyzed by using GraphPad Prism data analysis program (GraphPad Software, Inc., San Diego, CA, USA). For the comparison of statistical significance between patients and normal subjects, Student Newman-Keuls *t*-test for unpaired data was used. For each group paired two tailed Student's "*t*" test was used to compare values of varies parameters

tested pre and post chemotherapy, the differences were considered statistically significant when *p* values were less than or equal 0.05.

RESULTS

Clinical features of patients with NHL group I (before start of treatment) and group II (after 4 cycles of chemotherapy) are illustrated in Table (1).

Table (1): Clinical features of patients with NHL group I and II.

Group / Clinical data	Group I (n = 30)		Group II (n = 28)	
	Number	%	Number	%
Lymphadenopathy	26	86.6	12	42.8
Splenomegaly	4	13.3	1	3.6
Hepatosplenomegaly	6	20	5	17.8
B symptoms	20	66.6	17	60.7

B symptoms: include drenching night sweats, unexplained fever more than 38C°, metabolic wasting more than 10% of body weight in the preceding 6 months.

Disease characteristics of patients with NHL group I (before start of treatment) and group II (after 4 cycles of chemotherapy) are presented in Table (2).

Only erythrocytic sedimentation rate (ESR) showed statistically significant increase in the first and the second hours measurements in groups I and II versus control group ($p<0.001$) Table (3). After receiving four cycles of chemotherapy statistical significant reduction ($p<0.001$) was observed in ESR (1st and 2nd hours) in group II when compared to group I (Table 3).

Bcl-2 serum levels in patients with NHL group I and II showed highly statistical significant increase ($p<0.001$) than that in the control group (246.7±6.88 and 206.85±8.1 versus 148.62±8.69 ng/ml). Also, mean Bcl-2 serum levels of patients with NHL group II had highly significant reduction ($p<0.001$) comparable to group I (206.85±8.1 ng/ml versus 246.7±6.88) (Table 4).

When outcome was considered patients with NHL who developed complete remission showed significant decrease ($p<0.01$) in serum level of Bcl-2 (201.75±8.68) after receiving four cycles of chemotherapy compared to that before therapy (251.25±10.47) while patients

with NHL who developed PR or NR showed insignificant changes in relation to their levels before therapy (Table 5, Fig. 1). Serum levels of Bcl-2 in patients who developed CR, PR or NR showed insignificant changes when compared to each other either before or after treatment (Table 5, Fig. 1).

Fas/APO-l levels were significantly very high in sera of patients with NHL before treatment (group I) and after treatment (group II) and both showed high statistical significant values ($p<0.001$) as compared with levels in the control (group III) with mean value ± SE of 9.676±0.53, 8.610±0.90 versus 2.528±0.71 ng/ml respectively (Table 6). Fas/APO-l in patients after treatment showed statistical significant elevation ($p<0.01$) among those who had CR than in those with PR or NR (16.44± 0.88, 9.52±2.99 and 10.47±1.5 ng/ml respectively), Table (7), Fig. (2).

Table (2): Different disease characteristics in NHL patient's group I and II.

Group Parameter	Group I (n = 30)		Group II (n = 28)			
	Number	%	CR	PR	NR	Total and % of total
Stage:						
I	9	30	5	1	4	10 (35.7%)
II	11	36.7	4	1	4	9 (32.1%)
III	9	30	3	1	4	8 (28.6%)
IV	1	3.3	0	1	0	1 (3.6%)
Grade:						
Low	4	13.3	2	0	2	4 (14.3)
Intermediate	23	76.7	10	2	10	22 (78.6)
High	3	10	0	2	0	2 (7.1%)
Histopathology:						
Diffuse	20	66.7	10	2	7	19 (67.9%)
Follicular	10	33.3	2	2	5	9 (32.1%)
Presentation:						
Nodal disease	26	86.7	9	4	12	25 (89.3%)
Extra nodal disease	4	13.3	3	0	0	3 (10.7)
Bone marrow infiltration						
Present	3	3.3	0	0	1	1 (3.6%)

CR: Complete remission   NR: No remission   PR: Partial remission

Table (3): Comparison of the total leucocytic counts, platelet counts, hemoglobin levels and erythrocytic sedimentation rate in patient groups I, II and controls.

Group Parameter	Group I (before treatment) N = 30	Group II (after treatment) N = 28	Group III (control) N = 10	*p*-value		
				I vs. II	I vs. III	II vs. III
TLC (x 10^9/L)	6.86±3.25	6.66±2.33	5.83±1.13	NS p=0.366	NS p=0.339	NS p=0.288
Hb (gm/dl)	12 .92±1.38	11.26±2.27	14.74±1.70	NS p=0.286	NS p=0.749	NS p=0.076
Platelets (x 10^9/L)	254.6±123.9	239.4±99.9	240.2±60.96	NS p=0.299	NS p=0.726	NS p=0.982
ESR (1st Hour) mm	36.3±22.97	19.25±13.9	7.1±1.52	*** $p<0.001$	*** $p<0.001$	*** $p<0.001$
ESR (2nd Hour) mm	61.3±34.06	33.25±23	12.5±1.5	*** $p<0.001$	*** $p<0.001$	*** $p<0.001$

NS: Non significant   ***: $p<0.001$ TLC= Total leucocytic count
Hb= Hemoglobin   ESR= Erythrocytic sedimentation rate

Table (4): Bcl-2 serum level in NHL before treatment (group I), NHL after treatment (group II) and controls (group III).

Group BCL-2 (ng/ml)	Group I (before treatment) N = 30	Group II (after treatment) N = 28	Group III (control) N = 10	p-value		
				I vs. II	I vs. III	II vs. III
Mean ± SE	246.7± 6.88	206.85± 8.1	148.62± 8.69	***	***	***

***: *p*<0.001

Table (5): Comparison between level of Bcl-2 before and after treatment in NHL patients in relation to response of treatment.

Response Subject	CR N = 12	PR N = 4	NR N = 12	Significance		
				CR vs. PR	CR vs. NR	PR vs. NR
Bcl-2 (ng/ml) Before treatment (mean ± SE)	251.25±10.47	235±18.38	244.66±10.6	NS	NS	NS
Bcl-2 (ng/ml) After treatment (mean ± SE)	201.75±8.68	203.25±32.63	213.16±14.33	NS	NS	NS

p>0.050 is not significant (NS)   PR: Partial remission   CR: Complete remission   NR: No remission

NS: *p*> 0.050 is not significant
***: *p*< 0.001
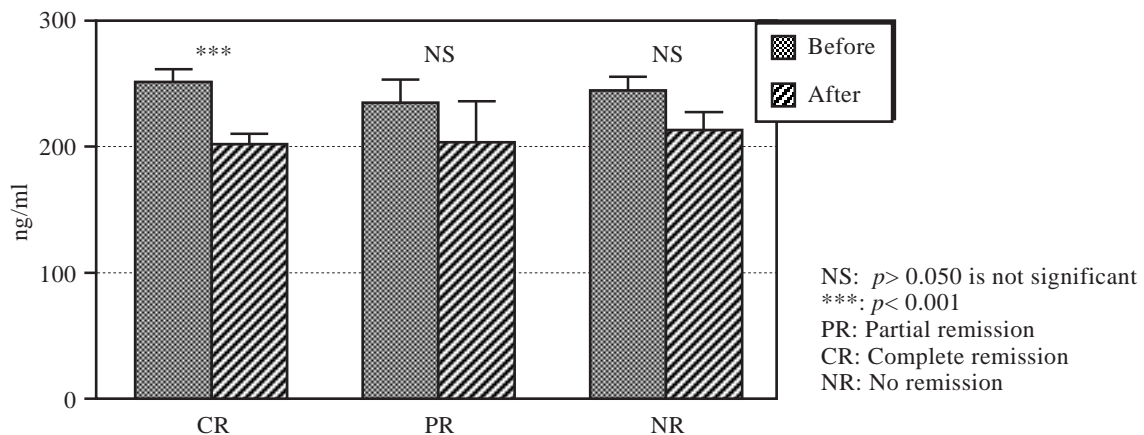PR: Partial remission
CR: Complete remission
NR: No remission

Fig. (1): Mean values ± SE of BCL-2 serum level before and after treatment in NHL patients in relation to response of treatment.

Table (6): Fas/APO-1(CD95) serum levels in patients with NHL before treatment (group I), after treatment (group II) and controls (group III).

Fas/APO-1 (CD95) ng/ml	Group I (before treatment) (n=30)	Group II (after treatment) (n=28)	Group III (control) (n=10)	p-value		
				I vs. II	I vs. III	I 1vs. III
Mean ± SE	9.676±0.53	8.610±0.90	2.528±0.71	NS	***	***

***: *p*< 0.001   NS: Non significant

Table (7): Comparison of serum levels of APO-1/FAS before and after treatment in relation to the response to treatment.

Response Subject	CR N = 12	PR N = 4	NR N = 12	Significance		
				CR vs. PR	CR vs. NR	PR vs. NR
Fas/APO-1 (ng/ml) Group I (Before treatment)	11.23±1.2	8.42±0.13	8.76±0.20	**	**	NS
Fas/APO-1 ng/ml Group II (After treatment)	16.44±1. 88	9.52±2.99	10.47±1.54	**	**	NS

NS= Non significant   **= *p*< 0.01   PR: Partial remission   CR: Complete remission   NR: No remission
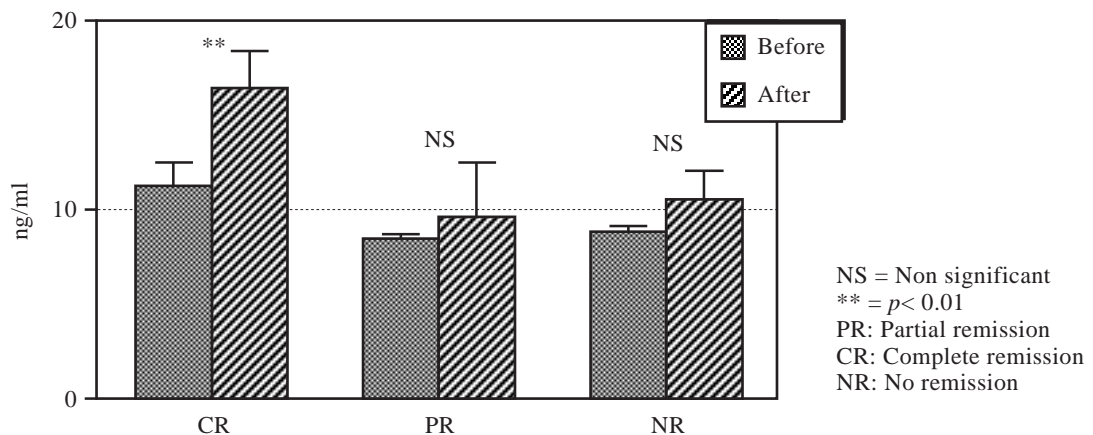
Fig. (2): Mean values of serum Fas/APO-1 in NHL patients before and after treatment with treatment outcome CR, PR and NR.

DISCUSSION

Some proteins regulating apoptosis as Bcl-2 and Fas/APO-1 (CD95) had been studied in 30 newly diagnosed patients with NHL. The clinical significance of Bcl-2 and Fas/APO-1 has been the subject of many studies [10,15,16,18, 19,20]. To our knowledge no clear-cut conclusions have been drawn regarding the prognostic importance of any of these parameters. This triggers us to conduct this study to clarify the interplay between apoptotic markers as Fas/APO-1 and proliferation markers as Bcl-2 with variable levels in various presentations in NHL, a finding that may be useful in predicting treatment outcomes.

The clinical characteristics of the patients included in this study were the predominance of peripheral lymphadenopathy in 86.6% of group I patients regressed to 42.8% after they completed 4 cycles of chemotherapy, hepatosplenomegaly in 20% reached only to 17.8% in patients group II. Also, 13.3% of patients group I had splenomegaly became only 3.6% after chemotherapy indicating that primary nodal and splenic infiltration responded more frequently to chemotherapy than extra nodal infiltration when the liver was affected. These data were consistent with that documented by Burkhardt et al.; Longo et al.; Yu et al. and Karadeniz et al. [7,21,22,23]. Also, B symptoms were present in 66.6% of patients group I reduced to 60.7% of group II patients, which is in agreement with results of Kamienska; Bakermerer et al.; Au et al. and Hwang et al. [5,24, 25,26].

Treatment outcome of our patients after completing 4 cycles of chemotherapy was: CR in 12 patients (40%), PR in 4 patients (13.34), NR in 12 patients (40%) with 2 defaulters (6.66%). This is consistent with the finding of Karadeniz et al.; Reed and Akhmedkhanov et al. [23,27,28] as they reported that almost all anticancer drugs available can kill tumor cells by activating endogenous biochemical pathways for cell suicide, known as programmed cell death or apoptosis. However, many malignant cells develop defects in the regulating genes that control the apoptotic pathway, thus rendering such cells more resistant to the used regimen of chemotherapy that may result in partial remission or no remission at all.

In relation to disease staging, there was increase in percentage of patients stage I and decrease in percentage of stages II and III after receiving chemotherapy indicating disappearance of one or more of lymph node groups with therapy without changes in extra nodal infiltration in stage IV. These findings are coinciding with those of Armitage and Weisenburger (1998), Marsden et al.; Salies and Na et al. [29-32].

Peripheral blood counts in the patient's groups were within normal ranges either at the start or after receiving four cycles of chemotherapy, which could indicate absence of bone marrow infiltration, present only in one patient. This disagrees with List et al. [33], who reported anemia in most of their patients at presentation secondary to lymphatic infiltration of bone marrow but coincided with data mentioned by Ansell et al. [34].

Erythrocytic sedimentation rate (ESR) showed highly significant higher values in patients with NHL both before and after receiving chemotherapy compared to control group due to disease effect. After receiving chemotherapy, highly significant reduction in ESR values in patients group II compared with group I indicating good response to treatment. These results matched with that of Banadonna and Ansell et al. **[17,34]**.

The Bcl-2 class of anti-apoptotic protein is an important inhibitor of the mitochondrial mediated pathway of apoptosis by preserving mitochondrial membrane integrity and preventing the release of cytochrome-C and other pro apoptotic molecules from the mitochondria to the cytosol or the nucleus **[30,35]**. Also, Bcl-2 has been suspected to be a major regulator of a homeostatic balance between cell survival and cell death, and can slow cell proliferation in both lymphocytes and myeloid cells **[11,36]**.

In the present study, Bcl-2 protein detected in sera of NHL patients before receiving chemotherapy was heterogeneous due to case to case variability (ranged from 197 to 316 ng/ml). However, the mean value was significantly higher in patients with NHL than that in control group (246.7 versus 148.62 ng/ml). This finding is consistent with that of Salem et al.; Tang et al.; Yunis et al. and Pezzela et al. **[8,10,37,38]** who reported that high levels of Bcl-2 protein was observed in follicular and diffuse non Hodgkin's Lymphoma.

In the current study, the mean values of Bcl-2 protein in sera of patients before treatment (group I) showed very high statistical significant level when compared to that after treatment (group II). This finding may explain the apoptotic effect of used chemotherapeutic agents, which was antagonized by the antiapoptotic Bcl-2 protein. This is consistent with the finding of Schmitt and Lowe **[39]** who found that high levels of initial Bcl-2 suppress apoptosis induced by depletion of survival factor, hypoxia and cytotoxic drugs. Also, Schendel et al. **[40]** suggested that Bcl-2 forms ion channels that allow the transport of an ion or a protein across the mitochondrial membrane in the direction that is presumably cytoprotective (anti-apoptotic). Alternatively Reed **[27]**, suggested that Bcl-2 forms cytotoxic channels and protects cells by nullifying the channel activity and thus promoting cell survival. On the contrary, **[11,12,41-44]** suggested that Bcl-2 has different anti-apoptotic functions depending on the level of Bcl-2 at the pro-apoptotic stages.

So, the function of Bcl-2 is dependent upon which other members of the Bcl-2 family that is dimerzes with or upon the phosphorylation status of Bcl-2 **[45,46]** and upon the upstream and downstream events **[47]**. The net effect of the Bcl-2 family may be pro-apoptotic despite the high level of Bcl-2 due to over expression of other pro-apoptotic members, or high Bcl-2 are a response to the stimulation of pro-apoptotic family members and that the cells need to maintain very high levels to prevent apoptosis.

These cells presumably are living on the edge of apoptosis, and because Bcl-2 expression is near its maximum, it cannot be raised further in response to an apoptotic signal, so the cell dies **[44,47]** in contrast that cell with lower Bcl-2 level do not require Bcl-2 for protection, and there is sufficient room for increases in Bcl-2 expression to occur in response to an apoptotic signal. Another possibility in those cells with lower levels may develop other methods of avoiding apoptosis (i.e. high expression of Bcl-XL), **[48]** or that downstream regulators of apoptosis are modified **[47]**.

In the current study there was no association between Bcl-2 expression and treatment outcome. This result is consistent with the study of Elbordini et al. **[49]**, who reported the same results. Another study by Reed **[50]** stated that elevation of Bcl-2 expression could cause drug resistance to chemotherapeutic agents, thus supporting the anti-apoptotic function of Bcl-2 while decrease in Bcl-2 expression promotes the apoptotic response to anticancer drugs.

An important factor of apoptosis in the immune system is Fas/APO-1 a synonym for CD95, a transmembrane receptor that is a member of the tumor necrosis factor and nerve growth factor receptor super family **[44,51]**.

In the present study Fas/APO-1 levels were significantly very high in sera of both NHL patients before treatment (Group I) and after 4 cycles of chemotherapy (Group II) than in group III (controls). These are consistent with the reports of Beltinger et al. **[52]** who found that Fas/APO-1 was detected in sera of patients with

human T-cell leukemia and was increased in the sera of patients with lymphoid malignancies and also in agreement with Xerri et al. and Zodelava & David [13,53] who reported that CD95 can trigger apoptosis in some lymphoid cell lines.

The report of AkhmedKhanov et al.; Kobayashi & Koike; Kono et al.; Kamihira & Yamada and Shimizu et al. [28,54-57] suggested that elevated Fas/APO-1 production may promotes tumor genesis and disease progression. In addition, Kondo and colleagues [57] reported that high CD95 levels had been detected in NHL patients also by immunohistochemical analysis. As well as the reports of Hara et al.; Reed & Pellecchia; Knipping et al. and Papoff et al. [14,44,59,60] who found that sera of patients with different high and low grade malignant B and T cell lymphomas had an increased levels of Fas/APO-1.

In this study, Fas/APO-1 in the sera of patient group II, showed significant statistical elevation among those who had CR than in those with PR or NR. This result is consistent with the study of Elbordini and Co-workers [49], who found that NHL patients who had high CD95 levels tended to be more sensitive to the used drugs and their patients acquired complete response than those with low levels. Also, interesting studies by Hara et al.; Lajmanovich et al.; Hazar et al.; Trauth et al.; Falk et al.; Aftabuddin et al.; Robertson et al. and Schattner et al. [14-16,61-65] in NHL patients with high levels of CD95 showed a higher apoptotic cell count than those with low levels of CD95 suggesting that Fas/APO-1 is involved in the apoptotic response of tumor cells to chemotherapy in NHL. In addition they reported that defect in apoptotic effect delivered through this antigen may contribute to the pathogenesis of hematological neoplasm.

So, from the results of this study we can conclude that increased serum levels of Bcl-2 in patients with NHL before treatment may indicate the possible anti-apoptotic role of Bcl-2 in tumor genesis, which antagonizes the effect of chemotherapy as inducer of apoptosis. Also, elevated levels of Fas/APO-1 is an important apoptotic marker in NHL, as it can reduce tumor genesis and tumor progression so it can be considered a good predictor for response to chemotherapy.

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